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[CANCER RESEARCH 55, 510–513, February 1, 1995]

Advances in Brief

Different Angiogenic Pathways Characterize Superficial and Invasive Bladder Cancer[1]

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Abstract

We have investigated by RNase protection analysis the expression of 2 angiogenic factors in 45 primary bladder tumors and 8 normal bladders.

Expression of vascular endothelial growth factor (VEGF) was 3-fold higher and that of platelet-derived endothelial cell growth factor was 40-fold higher in tumors compared to normal bladder. However, the factors were differentially expressed in different stages of the cancer. Expression of VEGF in superficial tumors was 4-fold higher than in invasive tumors and 10-fold higher than in normal bladder (superficial *versus* invasive, $P < 0.0006$; superficial *versus* normal, $P < 0.0002$). Expression of platelet-derived endothelial cell growth factor in invasive tumors was 33-fold higher than in superficial tumors and 260-fold higher than in normal bladder (invasive *versus* superficial, $P < 0.0001$; invasive *versus* normal, $P < 0.0003$).

In well differentiated or moderately differentiated superficial tumors which had invaded the lamina propria (pT1G1/2) VEGF expression was 4-fold higher in tumors which subsequently recurred at 3 months compared to those which did not recur ($P < 0.002$).

Thus there are two distinct angiogenic pathways involved in different stages of bladder cancer, which is in keeping with the evidence for two different genetic pathways. Elevated VEGF expression predicts early recurrence of pT1G1/2 tumors.

Introduction

Angiogenesis is a prerequisite for tumor growth and metastasis (1) and is induced by angiogenic factors produced by the tumor or the nonmalignant cells which infiltrate the tumor. Increased vascular density has been shown to correlate with a higher incidence of metastases and a worse prognosis in tumors of the bladder (2), breast (3, 4), skin (5), and prostate (6, 7). Numerous angiogenic factors have been described (8, 9). The relative importance of individual angiogenic factors in most tumor types is still largely unclear although differential angiogenic factor expression has been reported in renal cell carcinoma (10).

Currently there are few reports concerning bladder cancer angiogenesis. Increased expression of acidic fibroblast growth factor in bladder cancers compared to normal bladder has been demonstrated by immunohistochemistry (11), and acidic fibroblast growth factor has been identified in the urine of patients with bladder cancer (12). Elevated levels of basic fibroblast growth factor have also been identified in the urine of patients with bladder cancer (13). Most recently, increased expression of VEGF[3] was reported in three bladder tumor samples (14).

One of the most studied angiogenic factors is vascular endothelial growth factor which is a specific mitogen for endothelial cells. VEGF promotes angiogenesis by stimulating capillary proliferation, migration, and permeability (15). Transfection of breast and cervical cancer lines with VEGF accelerates tumor growth and increases tumor vascularity after implantation in nude mice (16, 17). Anti-VEGF antibodies have been shown to slow the growth of xenografted glioblastomas, leiomyosarcomas, and rhabdomyosarcomas in mice (18). PDECGF is a potent angiogenic factor (19) and has recently been shown to be thymidine phosphorylase (20, 21). PDECGF/TP is elevated in several solid tumor types (22–25). The mechanism of its angiogenic action is still unclear but is dependent on its enzymatic action (26). TP hydrolysis of thymidine gives rise to 2-deoxyribose 6-phosphate which is readily dephosphorylated to 2-deoxyribose. The latter has been reported to be angiogenic (27).

Bladder tumors fall into two main pathological categories, superficial and invasive, with quite distinct morphologies and behavior (28, 29). Invasive tumors (pT2, -3, -4) penetrate detrusor muscle and are usually solid and poorly differentiated (G3). Superficial tumors do not invade muscle, usually have a papillary morphology with a highly organized branching vasculature, and are typically well (G1) or moderately (G2) differentiated. Superficial tumors are further subdivided into pTa and pT1 lesions: pT1 tumors penetrate into the lamina propria; whereas pTa tumors do not. Stage progression occurs in only around 15–20% of superficial tumors but over 70% will recur despite treatment with surgery and/or intravesical chemotherapy. The molecular determinants of recurrence and stage progression are still incompletely understood (30).

We have determined the expression of VEGF and PDECGF/TP in a series of bladder cancers to ascertain whether differences in tumor phenotype and patterns of progression and recurrence might be associated with differential angiogenic growth factor expression.

Materials and Methods

Preparation of RNA and Construction of Plasmids to Generate Probes for RNase Protection Analysis

RNA was prepared from 45 primary bladder tumors and 8 normal bladder samples using the method of Chomczynski and Sacchi (31). The tumor samples were all obtained by transurethral resection of newly diagnosed tumors. None of the patients had been treated previously with radiotherapy or chemotherapy. The normal bladder samples were obtained at the time of donor nephroureterectomy prior to renal transplantation.

PDECGF/TP. Plasmid pPL5 incorporating the full-length cDNA of platelet-derived endothelial cell growth factor was digested with *Nco*I. The 5′ overhangs were end-filled by use of DNA polymerase I (Klenow fragment), and the plasmid was then digested with *Bam*HI. A 241-base pair fragment corresponding to 817–1058 nucleotides of the coding region of PDECGF was generated. The fragment was then cloned into the *Eco*RV/*Hind*III site prior to generation of radiolabeled antisense transcripts with T3 RNA polymerase.

VEGF. Plasmid pBluescript KS$^+$ containing the full-length cDNA of $VEGF_{121}$ was linearized with *Eco*RV, and a 520-nucleotide antisense fragment was generated with T7 polymerase. This fragment comprised full-length an-

Received 12/13/94; accepted 12/28/94.

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[1] T. O. was supported by The Private Patients Plan Research Scholarship of the Royal College of Surgeons of England. S. F. is supported by the Medical Research Council. R. B. and A. H. are supported by The Imperial Cancer Research Fund.

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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; PDECGF, platelet-derived endothelial cell growth factor; TP, thymidine phosphorylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

tisense $VEGF_{121}$ (363 nucleotides code for the mature protein and 81 nucleotides for the secretion peptide), antisense 5′-untranslated sequence (66 nucleotides), and 10 nucleotides of antisense vector. Alternative splicing of VEGF occurs at base pair 345 (32); thus with the 81 nucleotides coding for the secretion peptide, $VEGF_{121}$ mRNA protects a 444-nucleotide fragment. $VEGF_{165}$ and $VEGF_{189}$ mRNAs both protect a 425-nucleotide fragment and thus run to a more distal position than the $VEGF_{121}$ fragment during gel electrophoresis.

RNase Protection Analysis

Antisense probes, labeled with a [^{32}P]dCTP, were hybridized to 10 mg of total cellular RNA, and the free unhybridized probe was removed by digestion with RNases A and T1. Protected fragments were analyzed by electrophoresis in 6% polyacrylamide/urea-sequencing gels followed by autoradiography. In each hybridization an antisense transcript, corresponding to GAPDH (33), was included as an internal control. The abundance of mRNA was quantified by scanning laser densitometry (Bio Image analyzer; Millipore, Bedford, MA) and signals were standardized to the GAPDH control to provide a measure of expression. Two positive controls from recurrent carcinomas were loaded onto each gel.

Statistical Analysis

The levels of expression of VEGF and PDECGF in normal bladder and tumors were compared using the Mann-Whitney *U* test (two tailed).

Results

Seven of the tumors were pTa lesions, 21 were pT1 lesions, and 17 were invasive (pT2, -3, -4). Two of the invasive tumors were predominantly squamous cell tumors with all other tumors being transitional cell in origin. All the pTa tumors had a papillary morphology; 16 of the pT1 tumors were papillary, 2 were solid, and 3 were mixed. Three of the invasive cancers had a papillary morphology and 14 were solid.

Representative protection assays for VEGF and PDECGF are shown in Fig. 1. VEGF levels are available on all the samples. Expression of VEGF was 3-fold higher in tumors than normal bladder ($P < 0.0004$). Expression of VEGF in superficial tumors was 10-fold higher than in normal bladder ($P < 0.0002$) and 4-fold higher than in invasive tumors ($P < 0.0006$). Expression in invasive tumors was only

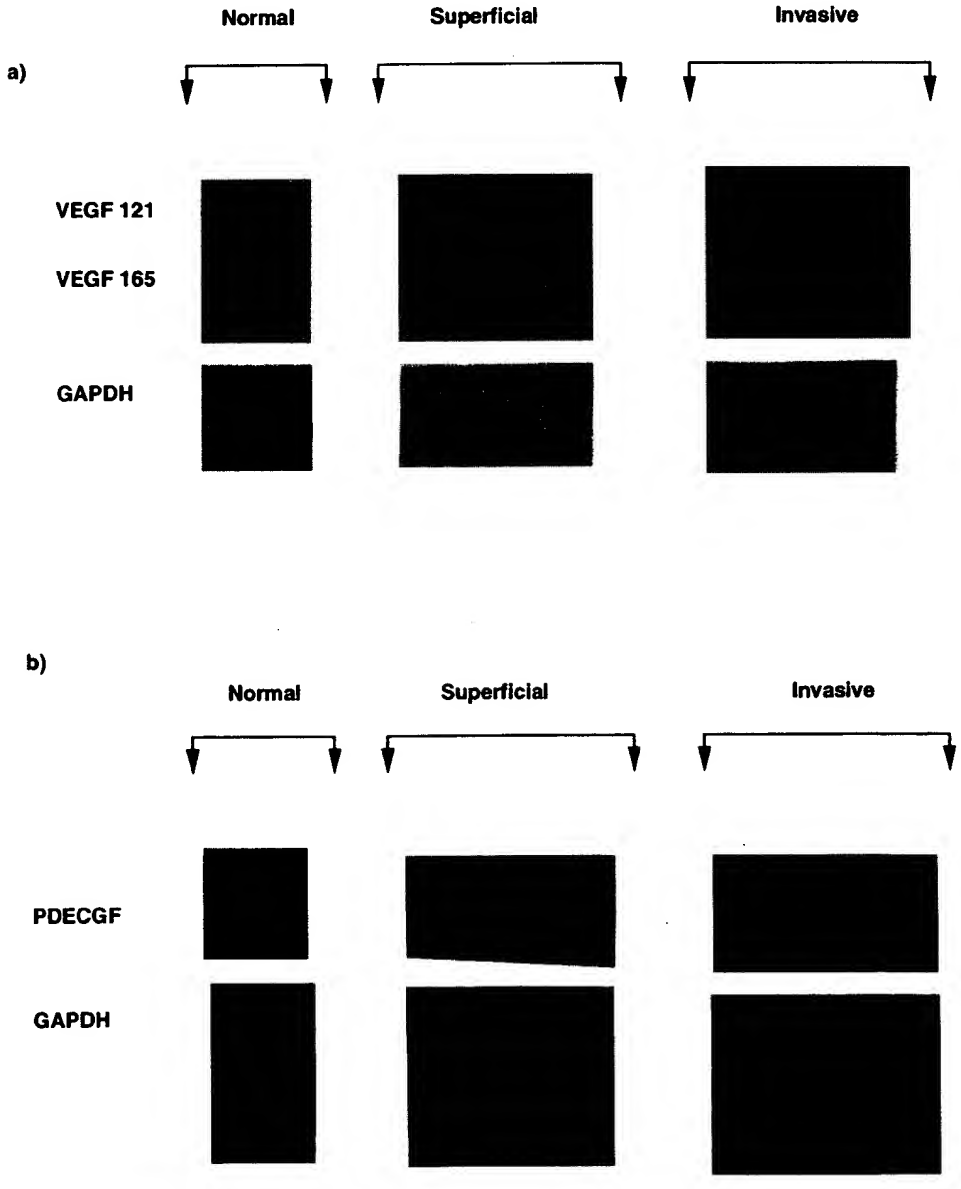
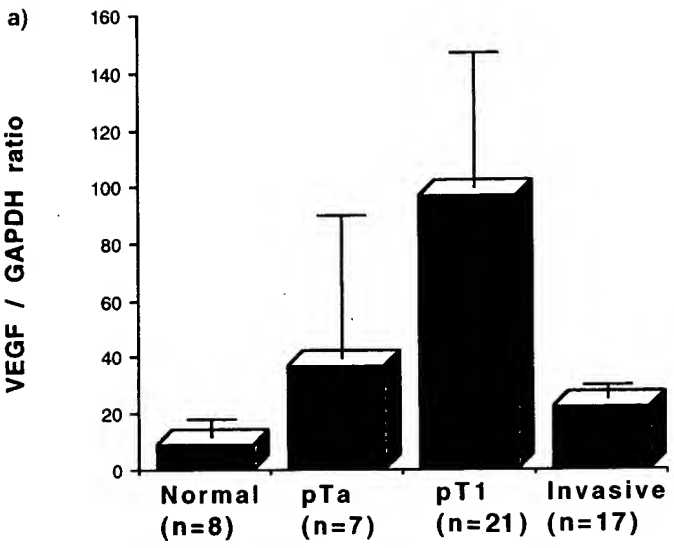


Fig. 1. RNase protection assays for VEGF (*a*) and PDECGF (*b*) in human bladder cancer. Tumor RNA (10 μg) was hybridized to a radiolabeled antisense probe specific for the VEGF or PDECGF mRNA and, after digestion with RNase, was electrophoresed in a 6% acrylamide gel. The $VEGF_{121}$ signal is seen to be stronger than the $VEGF_{165}$ signal. Quantitation of expression for each tumor was achieved by calculating the ratio of the absorbance of the angiogenic molecule ($VEGF_{121}$ or PDECGF) to the absorbance of the GAPDH signal in order to allow for loading inequalities.



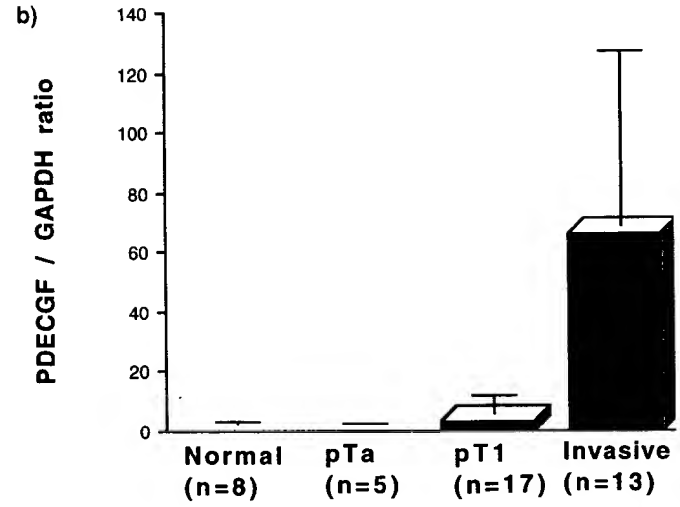


Fig. 2. Quantitated median levels of VEGF (*a*) and PDECGF (*b*) mRNA in bladder tumors of different pathological stages. Quantitation was achieved by laser densitometric scanning of autoradiographs. In each case the relative signal was calculated by ascribing an arbitrary value of 100 to the signal from a recurrent tumor which acted as a positive control and which was electrophoresed on all gels to allow cross-gel comparisons. *Bars*, 25–75 percentiles.

2-fold higher than in normal bladder ($P < 0.001$). VEGF expression was highest in the pT1 subgroup of superficial tumors (Fig. 2*a*).

PDECGF analyses were performed on 43 of the samples (8 normals and 35 tumors). Median PDECGF expression was 40-fold higher in tumors than normal bladder ($P < 0.01$). There was no statistically significant difference in expression between superficial tumors and normal bladder. Expression in invasive tumors was 260-fold higher than in normal bladder ($P < 0.0003$) and 33-fold higher than in superficial tumors ($P < 0.0001$) (Fig. 2*b*).

Eighteen of the 19 patients with pT1G1 or pT1G2 tumors underwent their first check cystoscopy at 3 months. Ten patients developed a recurrence. The VEGF/GAPDH level was 4-fold higher in those who subsequently recurred (median, 159; range, 95–420) than in those who did not recur (median, 36; range, 1–119) ($P < 0.002$) (Fig. 3). At a decision value of 95 the sensitivity of an elevated VEGF for predicting future recurrence is 100% and specificity is 88%.

Discussion

This study has shown that the angiogenic growth factors VEGF and PDECGF are up-regulated in bladder cancer but are differentially expressed; VEGF expression is higher in superficial tumors than invasive tumors but the reverse is true for PDECGF. This is the first demonstration of differential angiogenic factor expression in bladder

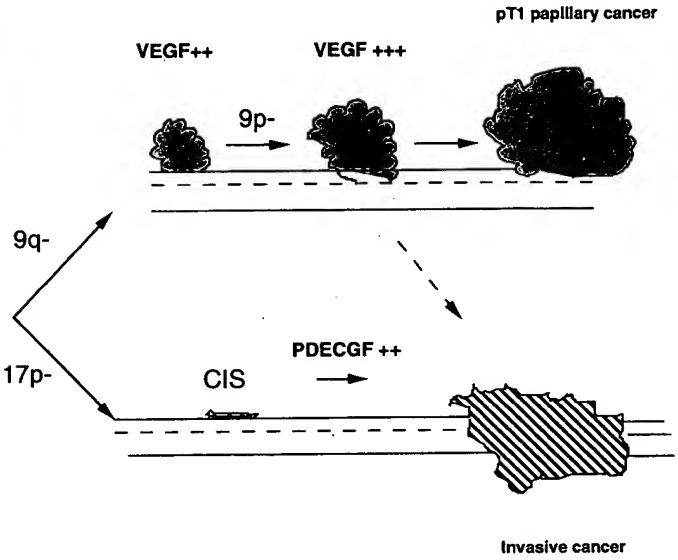


Fig. 4. Postulated genetic and angiogenic pathways in bladder cancer. Mutations of chromosome 9q are seen in up to 80% of pTa tumors but are rare in invasive tumors. We propose that VEGF is the key angiogenic molecule in the development of papillary lesions (*upper pathway*) and that markedly elevated VEGF expression leads to a more aggressive phenotype in this group of tumors. In the lower pathway the invasive tumors probably develop from carcinoma *in situ* (*CIS*). Mutations of 17p are seen in approximately 60% of these tumors. PDECGF is markedly elevated in invasive tumors and our hypothesis is that elevated expression may facilitate progression from carcinoma *in situ* to invasive disease.

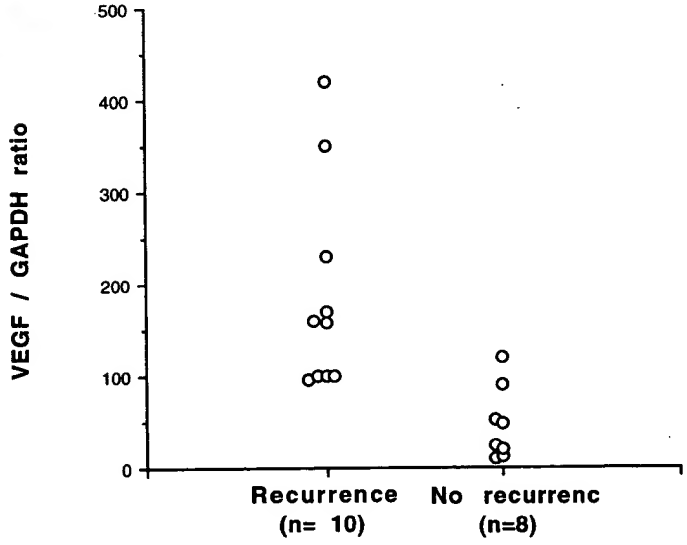


Fig. 3. Relationship between $VEGF_{121}$ mRNA expression in newly diagnosed pT1 G1/2 bladder tumors and tumor recurrence at 3 months. In each case the relative signal of $VEGF_{121}$ was calculated by ascribing an arbitrary value of 100 to the signal from a recurrent pT1G2 bladder tumor which acted as a positive control. Eighteen patients underwent a check cystoscopy of whom 10 developed a recurrence as judged by the macroscopic appearance at cystoscopy.

cancer. Superficial bladder tumors usually have a papillary morphology with a well organized branching vascular tree whereas invasive tumors are generally solid with a disorganized vasculature and areas of necrosis. Our results suggest that there may be two different angiogenic pathways in bladder cancer which are associated with different tumor morphologies and behavior (Fig. 4). Recent evidence has shown that there are two distinct molecular genetic pathways in bladder cancer (34–37) (Fig. 4). Approximately 70% of superficial tumors are thought to have mutations of 9p or 9q whereas mutations on chromosome 9 are more unusual in invasive tumors. Mutations of 17p are common in invasive cancers or carcinoma *in situ* but are rare in superficial tumors. These molecular genetic findings concur with the established clinical evidence that most invasive cancers of the bladder do not have a clearly defined superficial growth phase. Our data suggest that two different angiogenic pathways are associated with the two molecular genetic pathways, although both pathways clearly require increased angiogenesis. It is possible that the morphology of bladder tumors may in part be determined by the nature of the angiogenic factor expressed.

Within the subgroup of superficial tumors expression of VEGF appears to be associated with a more aggressive phenotype; an elevated VEGF was associated with a higher chance of recurrence of pT1G1/G2 tumors. Recurrent bladder tumors are thought to arise by implantation of tumor cells (38) from the original tumor. It is possible that, following implantation, the cells which express high levels of VEGF can establish a blood supply and grow more easily. As yet no tumors in this series have undergone stage progression but it will be of interest to see if VEGF expression is associated with stage progression in these patients.

Angiogenesis occurs in few physiological states (8); consequently inhibitors of angiogenesis are potentially promising novel agents in cancer therapy. Several of these inhibitors have been described (39) and inhibition of recurrence of superficial bladder cancer may be a suitable model by which to study their effects. Invasive bladder cancer currently carries a 50% mortality, however treated, so that new therapeutic approaches are required. The dramatic up-regulation of PDECGF in invasive bladder cancer identifies it as a therapeutic target. PDECGF activates 5′-deoxy-5-fluorouridine (Furtulon) which is a prodrug of 5-fluorouracil, and given the 260-fold increase in expression of PDECGF in invasive tumors Furtulon may be worthy of study in invasive bladder cancer.

Acknowledgments

We acknowledge the help of all of the surgeons and nurses of the Urology Service and the ICRF research nurses in Oxford.

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